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STUDIES ON THE EFFECT OF METHOTREXATE ON AMINO ACID INCORPORATION IN THE EPIDERMIS OF THE YOUNG RAT*

TREVOR W. E. ROBINSON, M.B., M.R.C.P.†

ABSTRACT

Studies on the effect of methotrexate on the uptake and distribution of tritiated amino acids in young rat epidermis are described. The drug given up to 72 hours before the tritiated precursors had no demonstrable effect on either the quantitative uptake using a new solubilization technique for scintillation counting, or on the qualitative distribution using autoradiography. There was no apparent effect over 24 hours if given shortly after the tritiated amino acids using autoradiography.

It is concluded that methotrexate has no gross effects on amino acid incorporation in young rat skin.

Methotrexate (MTX) is now widely used in the treatment of psoriasis, but the mechanism by which it produces temporary remission of the psoriatic lesion remains uncertain. It will depress mitosis in psoriatic and normal human epidermis (1, 2), and in the skin of rodents (3). It has been suggested that this effect on mitosis is responsible for remissions in psoriasis, (1, 2, 4) but there is some evidence that MTX may also affect the differentiating cell mass, as the production of enzymes necessary for carbohydrate metabolism are depressed by MTX (5). In addition, it has recently been suggested that topical MTX may rapidly affect differentiating cells, as judged by the rapid reappearance of the granular layer in treated psoriatic lesions (6).

Tritium (3H) labeled amino acids have been shown to be incorporated differentially in rat and human epidermis using autoradiography (7, 8) and this label is known to be due to newly synthesized protein (9, 10). In the present studies similar methods were undertaken to further investigate the amino acid incorporation of normal differentiating epidermal cells in young rat epidermis, and to ascertain whether MTX could be shown to have a qualitative effect on the differential grain distribution of 3H amino acids in the epidermal layers using autoradiography. In addition, quantitative studies on the epidermal uptake of similar precursors were undertaken, using a scintillation counter.

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Reprint requests to: R. B. Stoughton, M.D. 476 Prospect Street, La Jolla, California 92037.

* From the Division of Dermatology, Scripps Clinic and Research Foundation, La Jolla, California.

† Present address: University College Hospital, London, W.C.1, England.

MATERIALS AND METHODS

The chemicals and sources are listed below:

3H glycine—2.1 c/mM: 3H histidine—5.1 c/mM: 3H leucine—15.0 c/mM:
3H methionine—161 mc/mM: 3H proline—1.5 c/mM: 3H tyrosine—1.0 c/mM:
all supplied by Schwarz BioResearch, Orangeburg, N.J.

Puromycin—Nutritional Biochemical Company, Cleveland, Ohio.

Methotrexate—Lederle, American Cyanamid Company, Pearl River, N.Y.

Throughout these studies, 4–6 day old Sprague-Dawley rats kept under constant environmental conditions were used. Animals were injected with MTX (0.5 to 5.0 mg/kg) on the ventral surface at varying periods from 72 hours to 1 hour before, or 1 hour after, injecting 3H labeled amino acids (15 μc in 0.05 ml) using the same technique. Great care was taken to discard any animals in which leakage of injected material occurred from the puncture wounds in order to minimize dose variation and contamination. Animals were sacrificed by ether poisoning in clean jars and skin specimens of the required size were removed from the dorsum of each animal with carefully cleaned surgical instruments.

Autoradiographic technique. (Based on technique from Division of Dermatology, University of California, San Francisco Medical Center.) Pieces of interscapular skin 0.5 cm square were fixed in buffered formalin overnight and trans-

ferred to 70% alcohol for storage. Specimens were paraffin embedded, sectioned five microns thick, and affixed to slides using gelatin in the water bath at 45°C, placed in a 60°C incubator for one hour, allowed to cool, deparaffinized in xylene, dehydrated in absolute alcohol and 95% alcohol twice respectively. After air drying overnight, slides were dip-coated in Kodak NTB-2 emulsion, placed in lite-tite drierite® containing boxes, and stored at 4°C for 14–28 days. Slides were developed with Kodak 19 developer for 5 minutes, dipped in 1% acetic acid rinse for 10 seconds, and placed in Edwal quick fix® for three minutes, before washing in tap water for 10 minutes, and distilled water for 10 seconds. All solutions during development were maintained at 18°C. After air drying overnight, slides were stained with hematoxylin and eosin, mounted, and a preliminary microscopic examination was carried out to insure that the autoradiographs were satisfactory before slides were coded for counting blind.

Grain counts in the malpighian layers, and the horny layer if indicated, were carried out using a Whipple's eyepiece micrometer disc under × 100 oil immersion lens (final magnification × 1000). The number of grains appearing in sections varied considerably, so a ratio of counts in 100 squares in each layer was computed, assigning the count in the basal cells as unity (8).

Tissue preparation and scintillation counting technique. Ten gram young rats were injected on the ventral surface with each 3H precursor and methotrexate as indicated previously, and killed with ether. Most of the dorsal skin of each animal was removed without including the subcutis. Skin specimens were immediately placed in carefully flattened, previously labeled aluminium foil envelopes, and the separation of epidermis from dermis was accomplished by placing the aluminium foil envelopes with dermis down on a slide warmer at 56°C for 30 seconds under a paper pad and staining jar on which light manual pressure was maintained. Complete epidermal separation was then easily accomplished using clean forceps (histological confirmation of clean separation was obtained).

Epidermal specimens (100–150 mg in weight) were thus obtained from each rat, and approximately 10 mg portions (exact weight recorded) were obtained for scintillation counting.

Washing consisted of two 15 minute immersions in 5% trichloracetic acid (TCAA) at room temperature, subsequent dipping in distilled water and drying on clean filter paper. This procedure was found to be adequate as insignificant counts were obtained from TCAA aliquots after the second wash.

Epidermal solubilization was then effected by using a modified technique as suggested by Beckman Co. (11). Epidermal specimens (10 mg) were digested in 0.5 ml of 2N NaOH in new scintillation counting bottles in an incubator at 80°C for 30 minutes, cooled to room temperature and 1.5 ml of Beckman Bio-Solv BBS_2 solubilizer was added to each bottle from a Cornwall pipette. Gentle shaking produced a clear solution, to which one drop of 0.5% stannous chloride was added before adding 10 ml of Fluoralloy Beckman scintillation cocktail. Specimens were then counted at ambient temperature in a Beckman LS 150 scintillation counter. The counting efficiency for tritium in solutions prepared in this manner was 30 per cent.

RESULTS

Grain counts over a given area of the epidermal layers in MTX and control animals using 6 different 3H-amino acids are recorded in Table I. The numbers indicate the ratio of grain counts in the upper layers as compared to that in the basal layer which is taken as one. It can be seen that intraperitoneal methotrexate (in a dose of 0.5 mg/kg 1 hour, and 2.0 mg/kg 12 hours) before 3H-precursors had no significant effect on the distribution of grains at 1 or 24 hours after the intraperitoneal administration of 3H-amino acids. In addition, it can be seen that glycine and histidine are incorporated mainly in the granular layer, leucine and methionine in the prickle and basal layers, and tyrosine about the same in all layers. By 24 hours, histidine produces a continuous label in the lower part of the horny layer.

The results of a series of experiments using subcutaneous methotrexate (5.0 mg/kg) at 24, 12, 3 and 1 hour intervals before administering 3H-histidine and 3H-leucine subcutaneously and sacrificing 1 hour later are recorded in Table II. The numbers are mean ratios obtained from grain counts from 3 or more animals in at least 3 separate experiments. Comparison of figures in each MTX group with those in water injected and normal unhandled animals shows that there is no significant difference in the groups investigated. In addition, Figure 1 is a diagrammatic representation of the mean ratios obtained from each experimental group, with each 3H-amino acid, and there is no significant difference between groups. Table III contains figures representing mean ratios of mean grain counts when normal animals were given 3H histidine and 3H leucine, followed an hour later by methotrexate 5 mg/kg subcutaneously, and sacrificed at 6 and 24 hours thereafter. Experiments were conducted in the same way as for data in Table II and, as can be seen in Table III, there were no significant differences noted between the different groups.

TABLE I

Distribution of grains in autoradiographs of young rat epidermis 1 and 24 hours after injection of tritium labeled amino acids in methotrexate treated and control animals

Methotrexate dosage	Tritium labeled amino acid	***Mean ratio of counts of 1-hr grain distribution						Mean ratio of counts of 24 hr grain distribution							
		Methotrexate treated			Control			Methotrexate treated				Control			
		Gran layer	Prickle layer	Basal layer	Gran layer	Prickle layer	Basal layer	Horny layer	Gran layer	Prickle layer	Basal layer	Horny layer	Gran layer	Prickle layer	Basal layer
0.5 mg/kg* per animal	glycine	3.0	1.1	1.0	2.8	1.3	1.0	—	1.6	1.0	1.0	—	1.6	1.2	1.0
	histidine	2.9	1.3	1.0	2.4	1.1	1.0	2.8	1.6	1.0	1.0	3.1	2.0	1.1	1.0
	leucine	0.8	1.1	1.0	0.6	0.8	1.0	—	0.6	0.7	1.0	—	0.6	0.9	1.0
	methionine	0.5	1.0	1.0	0.4	0.9	1.0	—	0.9	1.1	1.0	—	0.9	1.1	1.0
	tyrosine	1.0	1.1	1.0	1.1	1.0	1.0	1.1	1.3	1.1	1.0	1.3	1.5	1.2	1.0
2.0 mg/kg** per animal	histidine	3.0	1.5	1.0	3.5	1.5	1.0	2.4	2.2	0.9	1.0	2.5	2.3	0.8	1.0
	leucine	0.7	0.8	1.0	0.5	0.8	1.0	—	0.8	0.9	1.0	—	0.8	1.0	1.0
	proline	0.9	1.0	1.0	0.9	0.9	1.0	—	0.9	0.7	1.0	—	1.2	0.9	1.0

* MTX given intraperitoneally 1 hour before amino acids.
** MTX given intraperitoneally 12 hours before amino acids.
*** Mean grain count from three animals for each amino acid, expressed as a ratio (see methods).

TABLE II

Distribution of grains of 3H-amino acids in autoradiographs of skin from young rats receiving preceding methotrexate

Amino acid	*Time of MTX injection	Mean ratio of mean grain counts in rat epidermis								
		** MTX injected group			Water injected control group			Normal unhandled control group		
		Granular	Prickle	Basal	Granular	Prickle	Basal	Granular	Prickle	Basal
3H histidine	24 hours	2.5	1.4	1.0	2.9	1.2	1.0			
	12 hours	2.6	1.4	1.0	2.6	1.2	1.0			
	3 hours	2.6	1.3	1.0	3.0	1.5	1.0	2.4	1.2	1.0
	1 hour	2.7	1.3	1.0	2.3	1.0	1.0			
	Mean	2.6	1.4	1.0	2.7	1.2	1.0			
3H leucine	24 hours	0.5	0.9	1.0	0.5	0.8	1.0			
	12 hours	0.6	1.0	1.0	0.5	1.0	1.0			
	3 hours	0.5	0.8	1.0	0.4	0.9	1.0	0.5	0.8	1.0
	1 hour	0.5	0.8	1.0	0.5	0.8	1.0			
	Mean	0.5	0.9	1.0	0.5	0.9	1.0			

* Time elapsing after injections before administration of 3H amino acids.
** MTX 5.0 mg/kg subcutaneously in 0.5 ml H_2O. Controls given H_2O or unhandled as indicated.

TABLE III

Distribution of grains of 3H-histidine and 3H-leucine in autoradiographs of skin of young rats given subsequent methotrexate and sacrificed thereafter

Amino acid	* Duration of MTX effect	Mean ratio of mean grain counts in epidermal tissue											
		** MTX treated group				H_2O injected control group				Normal unhandled group			
		Horny	Granular	Prickle	Basal	Horny	Granular	Prickle	Basal	Horny	Granular	Prickle	Basal
3H histidine	6 hour	—	2.2	1.2	1.0	—	2.1	1.3	1.0	—	2.2	1.3	1.0
	24 hour	3.3	3.1	1.1	1.0	4.2	2.4	1.4	1.0	3.4	2.6	1.4	1.0
3H leucine	6 hour	—	0.4	0.9	1.0	—	0.4	0.5	1.0		Not done		
	24 hour	—	0.7	0.9	1.0	—	0.5	0.9	1.0				

* Time interval between MTX administration and subsequent sacrifice.

** Methotrexate 5.0 mg/kg subcutaneously in 0.05 ml H_2O given 1 hour after 3H-amino acids. Controls given H_2O only or unhandled as indicated.

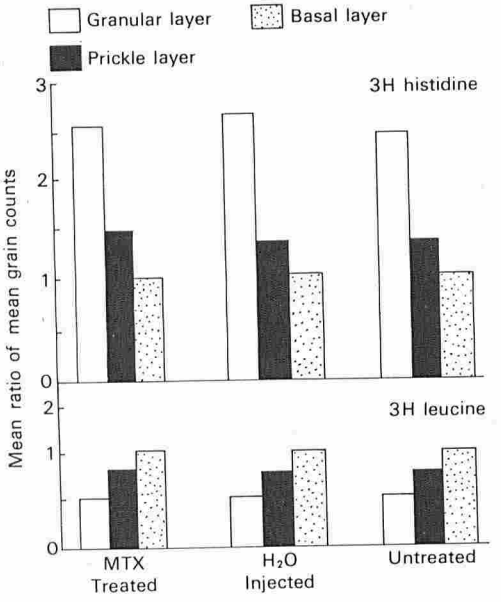

FIG. 1. Diagram of the distribution of grains of 3H histidine and 3H leucine in the epidermis of the young rat in MTX treated and control animals.

The DPM/mg (disintegration per minute per milligram) of 3H-histidine and 3H-leucine incorporated in epidermal tissue from MTX (5 mg/kg at 72, 48, and 24 hours before 3H-amino acids) and water injected animals are recorded in Tables IV and V respectively; it can be seen that there is no significant difference in uptake between experimental or control animals with either of the 3H-amino acids.

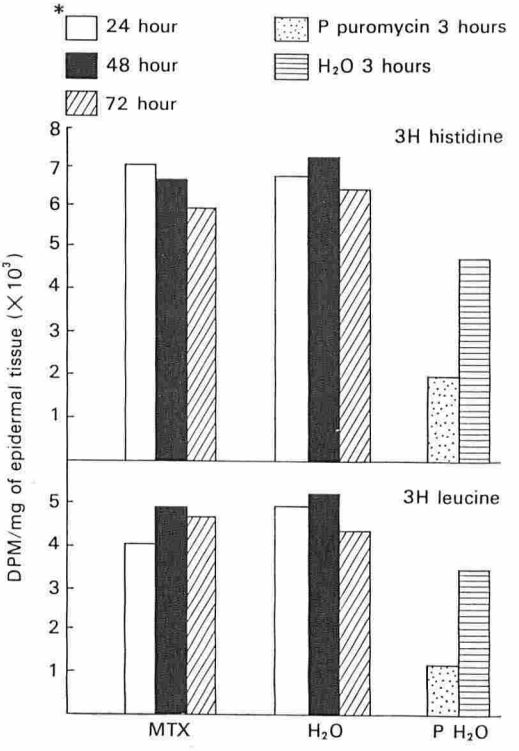

FIG. 2. Effect of MTX and puromycin on the incorporation of tritium labeled amino acids into epidermal cells.

These figures were derived from the mean counts on three separate pieces of tissue from each animal in each group; there were at

least five animals per group, and a minimum of two experiments were performed. Counts per minute per milligram rarely varied by more than 15%. Results from Tables IV and V are represented diagrammatically in Figure 2, alongside results from similar experiments in which the effect of puromycin was investigated; detailed results of this work are to be published elsewhere (12), but puromycin can be seen to produce rapid and profound depression of incorporation of the radioactive amino acids, in contrast to methotrexate. In the previous publication (12), proteins were extracted by two methods and the details are stated in that paper. The more extensive extraction procedure was also carried out in this series of animals and, as in the previous report (12) the differences between the two procedures prove to be of the same order in treated and untreated groups of animals.

TABLE IV

DPM/mg of 3H-histidine in young rat epidermis in methotrexate treated and control animals

	Mean DPM/mg of epidermal tissue	
* Time of injections	MTX treated (5 mg/kg)	Water injected control
24 hr.	7062	6930
48 hr.	6935	6990
72 hr.	5958	6468

* Time elapsing after injections before administration of 3H-histidine.

TABLE V

DPM/mg of 3H-leucine in young rat epidermis in methotrexate treated and control animals

	Mean DPM/mg of epidermal tissue	
* Time of injections	MTX treated (5 mg/kg)	Water injected control
24 hr.	3782	4051
48 hr.	4659	5043
72 hr.	4498	4193

* Time elapsing after injection before administration of 3H-leucine.

DISCUSSION

The autoradiographic studies confirm that differential incorporation of 3H-amino acids occurs in mammalian epidermis, as previously described by other workers (7, 8). In addition, MTX has not been shown to have any demonstrable qualitative effect on this pattern of incorporation if given at intervals before (Tables I and II and Fig. I), or just after (Table III), the radioactive precursors. As the autoradiographic epidermal label by 3H-amino acids has been shown to be due to newly synthesized protein, (9, 10) it would appear that MTX as used in these studies has produced no discernible qualitative effect on epidermal protein synthesis involving these amino acid precursors.

Likewise, quantitative studies using a modified solubilization technique have failed to demonstrate a significant depression of incorporation of radioactive histidine or leucine (Table IV) by MTX (5 mg/kg) given subcutaneously up to 72 hours before administration of the radioactive precursors, but puromycin produced a 50–75% depression of incorporation of these amino acids in young rat epidermis using these methods (12). As it is known that counts may be associated with lipoproteins and nucleic acids, these methods demonstrate a quantitative depression of epidermal amino acid uptake induced by puromycin that is not paralleled by methotrexate, but this may or may not represent an effect on true protein synthesis.

The lack of folate coenzymes could lead to failure of DNA, RNA, and protein synthesis. The folate coenzymes have been shown to be necessary in formylmethionine biosynthesis (13), and formylmethionine-S-RNA is the initiator of protein synthesis in bacteria (14), and is also responsible for some protein synthesis in Hela cells in tissue culture (15). In addition, MTX can affect mitochondria (15), and it has been shown to produce depression of certain enzymes necessary for carbohydrate metabolism in human epidermis (5). Theoretically, therefore, it would not be surprising if methotrexate were to produce effects on differentiating epidermal cells, including the incorporation and distribution of amino acid precursors. Robinson and Stoughton (3) have shown that in comparable concurrent experiments methotrexate rapidly depresses the

mitotic counts in young rat epidermis, and so it seems likely that active MTX was reaching the epidermis in these studies. However, no effects on amino acid uptake or distribution have been demonstrated with the techniques used in this work.

REFERENCES

1. Fry, L. and McMinn, R. M. H.: Action of methotrexate on skin and intestinal epithelium in psoriasis. Arch. Derm., *93:* 726, 1966.
2. Biro, L., Carriere, R., Frank, L., Minkowitz, S. and Petrou, P.: Morphologic changes induced by methotrexate. Histologic studies of normal and psoriatic epidermis. J. Invest. Derm., *48:* 429, 1967.
3. Robinson, T. W. E. and Stoughton, R. B.: The effect of methotrexate on cell division in the epidermis of the young rat. J. Invest. Derm., *53:* 223, 1969.
4. Van Scott, E. J. and Reinertson, R. P.: Morphologic and physiologic effects of chemotherapeutic agents in psoriasis. J. Invest. Derm., *33:* 357, 1959.
5. Halprin, K. M. and Ohkwara, A.: Methotrexate. Effect on the enzymes of carbohydrate metabolism in psoriasis. Arch. Derm., *96:* 565, 1967.
6. Fry, L. and McMinn, R. M. H.: The action of chemotherapeutic agents on psoriatic epidermis. Brit. J. Derm., *80:* 373, 1968.
7. Fukuyama, K. F., Nakamura, T. and Bernstein, I. A.: Differentially localised incorporation of amino acids in relation to epidermal keratinization in the new-born rat. Anat. Rec., *152:* 525, 1965.
8. Fukuyama, K. F. and Epstein, W. L.: Epidermal keratinization: localisation of isotopically labelled amino acids. J. Invest. Derm., *47:* 551, 1967.
9. Droz, B. and Warshawsky, H.: Reliability of the radioautographic technique of newly synthesized protein. J. Histochem. Cytochem., *11:* 426, 1963.
10. Fukuyama, K. F. and Epstein, W. L.: Ultrastructural autoradiographic studies of keratohyalin granule formation. J. Invest. Derm., *49:* 595, 1967.
11. Budd, A. L., Newman, F. and Thompson, B.: Liquid scintillation counting of aqueous solutions. Beckman Product Information. Bulletin No. 800. Part No. 80800.
12. Stoughton, R. B. and Robinson, T. W. E.: Effect of puromycin on mitosis and DNA synthesis of epidermal cells. J. Invest. Derm., *54:* 405, 1970.
13. O'Brien, J. S.: The role of folate coenzymes in cellular division. A Review. Cancer Res., *22:* 267, 1962.
14. Guthrie, C. and Nomura, M.: Initiation of protein synthesis. A critical test of the 30 S subunit model. Nature, *219:* 232, 1968.
15. Galper, J. and Darnel, J.: The presence of N-formylmethionyl-t-RNA in Hela cell mitochondria. Biochem. Biophys. Res. Comm., *34:* 205, 1969.